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Processing of Human Intestinal Prolactase to an Intermediate Form by Furin or by a Furin-like Proprotein Convertase*

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Human lactase-phlorizin hydrolase (human-LPH) is synthesized as a large precursor (prepro-LPH), then cleaved to a pro-LPH of 220 kDa which is further cut to a "mature-like LPH" of a size close to that of mature LPH, *i.e.* about 150 kDa (in the processing of rabbit pro-LPH the intermediate has a mass of approximately 180 kDa). By coexpression of human prepro-LPH with furin in COS-7 cells we show that furin generates a mature-like LPH. Radioactive amino acid sequence analysis reveals that furin recognizes the motif R-T-P-R^{832}, a protein convertase consensus, to generate a NH_2 terminus located 36 amino acids upstream of the NH_2 terminal found *in vivo* at Ala^{869}. This intermediate is ultimately cleaved to the mature LPH form by other proteases including the pancreatic ones. These data demonstrate that human pro-LPH, like the rabbit enzyme, is processed to the mature enzyme by furin or furin-like enzymes through at least an intermediate form that has, however, an apparent mass close to that of the mature enzyme.

Small intestinal lactase-phlorizin hydrolase (LPH,[1] EC 3.2.1.23–62), hydrolyzing lactose and other β-glycosides, is an integral membrane glycoprotein located in the intestinal brush-border membrane. Human LPH is synthesized as a large precursor (prepro-LPH) of 1927 amino acid residues, beginning with the leading sequence followed by four homologous regions (I–IV) and then by a membrane-spanning domain (1, 2) and a cytosolic COOH-terminal sequence (3). This precursor is glycosylated and subjected to proteolytic processing events on its way from the endoplasmic reticulum (ER) to the brush-border membrane. Mature LPH consists of somewhat more than the COOH-terminal half portion of prepro-LPH, *i.e.* two of the four homologous regions (regions III and IV) plus the transmembrane and the cytosolic segments.

The first protease acting on prepro-LPH is signal peptidase in the ER, which was predicted (1) and found in transfected MDCK cells (4) to split the human prepro enzyme between positions 19 and 20. Subsequently, enterocytes process pro-LPH to a "mature-like" LPH apparently in one step (5–8), whereas in the rabbit the formation of a 180-kDa intermediate is clearly evident (9, 10). Note that we indicate as *mature-like* LPHs those lactase forms that have a molecular size similar to, but have not been demonstrated to have an amino acid sequence identical to that of mature brush-border LPH.

Endoproteolytic cleavages at sites comprising the sequence motifs -R-R- or K/R-*X*-*X*-R- participate in the post-translational processing of many proteins. The subtilisin-related proprotein convertases (PCs) are a family of calcium-dependent serine endoproteases. These enzymes share between 50 and 74% amino acid sequence identity in the subtilisin-like catalytic domain (for reviews on their substrate consensus motifs, see, *e.g.*, Refs. 11 and 12). Two main subsets of enzymes can be distinguished. The first includes convertases such as PC2 (13) and PC1/3 (14), which are expressed mainly in neuroendocrine tissues and brain, in which they are located in secretory vesicles. These enzymes play a key role in allowing the formation of active hormones or neuropeptides from biologically inactive precursors. The second subset of convertases, which includes furin (15, 16), PACE4 (17), and PC5/6 (18, 19), shows wide tissue expression in the body. PACE4, PC5/6B, and especially furin have been shown to be involved in precursor processing of constitutively secreted proteins such as growth factors and their receptors (20, 21) and in cleavage of viral proteins (22). mRNAs of furin, PC1/3, and PC6A have been detected in rabbit enterocytes by *in situ* hybridization (23).

Recent reports have suggested that processing of human pro-LPH to mature LPH in the intestine occurs in more than one step (4, 24). The detailed mechanism, however, is not known. Because the pro region of human LPH contains several potential cleavage sites for subtilisin-like proteases (one site occurs upstream of, but not immediately preceding the NH_2 terminus of mature, brush-border LPH), we examined their potential to process human pro-LPH. We have now identified furin as a likely candidate involved in the first step of the maturation of human pro-LPH. Furin or furin-like PC(s) can generate a mature-like LPH that is 36 amino acids longer than mature LPH. Further proteolytic processing of this mature-like LPH form of human lactase to generate the final mature LPH form includes the eventual, albeit limited, action of pancreatic proteases (*e.g.* trypsin).

EXPERIMENTAL PROCEDURES

Materials—All chemicals were of the highest possible purity and were purchased from Fluka (Buchs, Switzerland) unless otherwise indicated. pSCTmLPH (human prepro-LPH (25)) was prepared in our laboratory. The cDNA coding for mouse furin, pSCTmfurin (26), was provided by Dr. K. Nakayama, University of Tsukuba, Japan, and subcloned into the vector pSCT Gal-X-556 (27).

Mutagenesis and Construction of cDNA Clones—Three double mutations were generated at basic residues located upstream of the NH_2 terminus of mature lactase: R829T/R832T, K853T/R854T, and K866T/R868T (see Fig. 4). Prepro-LPH cDNA was subcloned from the plasmid

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[1] The abbreviations used are: LPH, lactase-phlorizin hydrolase; ER, endoplasmic reticulum; MDCK cells, Madin-Darby canine kidney cells; PC, proprotein convertase; wt, wild type; BES, *N*,*N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid; PBS, phosphate-buffered saline; BFA, brefeldin A; PNGase F, peptide-N-glycosidase F; PAGE, polyacrylamide gel electrophoresis.

pSCTmLPH (human prepro-LPH (25)) into the vector pGEM-2 (Promega, Switzerland). A 3.5-kilobase fragment was deleted with *Pvu*II and the resulting plasmid used for site-directed mutagenesis using the Unique Site Elimination mutagenesis kit (Pharmacia, Switzerland). The mutagenesis reactions were performed with the following mutant sense primers (the nucleotides causing the mutation are underlined): LPH R829T/R832T, 5′-GC AGC AAG TCA ACG ACT CCC ACG AAA TCT GCC-3′; LPH K853T/R854T, 5′-CC AAG GGG GCA ACA ACA CTG CTA CCA CC-3′; LPH K866T/R868T, 5′-C CTC CCC TCC ACA GTC ACA GCC TTC ACT TTT CC-3′. The mutations were confirmed by DNA sequencing using the following sense primer: 5′-CCT TCT GGT TAC AGC CAG CGG-3′. After mutation, the 3.5-kilobase *Pvu*II fragment was reinserted. The final expression vectors, pcDNA I/Amp LPH R829T/R832T, pcDNA I/Amp LPH K853T/R854T, pcDNA I/Amp LPH K866T/R868T, and pcDNA I/Amp LPH wt (wild type), were constructed by cloning the mutated and wt LPH cDNA into the *Eco*RI site of pcDNA I/Amp (Invitrogen, Switzerland).

Transient Transfection of Cells—COS-7 cells, cultured in Dulbecco's modified Eagle's medium and 10% fetal calf serum (Life Technologies, Switzerland), were transiently transfected as described by Chen and Okayama (28). One day before transfection, the cells were seeded into six-well plates, to reach approximately 80% confluence the next day. During the last 30 min before transfection, the cells were incubated in 1.5 ml of fresh Dulbecco's modified Eagle's medium and 10% fetal calf serum at 37 °C and 3.5% CO_2. The calcium phosphate-DNA mixture was prepared by mixing 5 μg of plasmid DNA with 100 μl of 250 mM $CaCl_2$ and 100 μl of 2 × BBS (50 mM BES, 280 mM NaCl, 1.5 mM Na_2HPO_4, pH adjusted with NaOH to exactly 6.95) and incubating for 15 min at room temperature. 150 μl of this mixture was then added slowly to the cells, and incubation was continued for 24 h at 37 °C and 3.5% CO_2. The following day, the cells were washed twice with phosphate-buffered saline (PBS), refed 2 ml of fresh medium, and incubated for 24 h at 37 °C and 5% CO_2.

Biosynthetic Labeling and Immunoprecipitation—For metabolic labeling studies, the cells were washed with minimal essential medium (methionine-free) 48 h after transfection and then incubated in 2 ml of methionine-free labeling medium (minimal essential medium supplemented with 10% dialyzed fetal calf serum) for 1 h at 37 °C and 5% CO_2. After depletion of methionine, the cells were pulse labeled in 1 ml of labeling medium containing 25 μCi of [^{35}S]methionine (Amersham, Switzerland, specific activity > 1,000 Ci/mmol) for the time period indicated. The cells were washed twice with PBS and chased in Dulbecco's modified Eagle's medium and 10% fetal calf serum supplemented with 10 mM methionine. In some experiments, brefeldin A (BFA) was added to all media at a concentration of 5 μg/ml. The furin inhibitor decanoyl-Arg-Val-Lys-Arg-chloromethyl ketone (29) was added to all media needed at a final concentration of 100 μM. The labeled cells were collected and solubilized in 400 μl of lysis buffer (25 mM Tris-HCl, pH 8.0, 50 mM NaCl, 1% deoxycholate, 1% Nonidet P-40, 0.01 volume of 100 mM phenylmethylsulfonyl fluoride, 0.01 volume of inhibitor mixture (0.25 mg/ml pepstatin, 0.06 mg/ml aprotinin, 1.1 mg/ml leupeptin, 4.7 mg/ml benzamidine, 0.24 mg/ml bestatin, 0.3 mg/ml E-64, and 38.3 mg/ml *o*-phenanthroline). Immunoprecipitations, using a monoclonal mouse anti-human LPH antibody (30), were performed as described by Lottaz *et al.* (31). In some experiments equal volumes of cell lysates were treated with 50 μg of trypsin for different time intervals at 37 °C, and then the reaction was stopped with phenylmethylsulfonyl fluoride, the inhibitor mixture, and 200 μg of soybean trypsin inhibitor. The samples were then processed further for immunoprecipitation.

N-Glycosidase F Treatment—Immunoprecipitated proteins were eluted from Sepharose beads by boiling in 30 μl of 100 mM sodium acetate, pH 5.5, containing 0.2% SDS for 4 min. Then 3 μl of 10 × Glyco F buffer (500 mM Tris-HCl, pH 6.8, 200 mM β-mercaptoethanol, 1% SDS, 5% Nonidet P40) and 200 milliunits of peptide-*N*-glycosidase F (PNGase F, Boehringer, Switzerland) were added, and the mixture was incubated for 16 h at 37 °C.

SDS-PAGE—Samples (immunoprecipitates and proteins subjected to PNGase F treatment) were analyzed by SDS-PAGE in 5% acrylamide gels according to Laemmli (32). The gels were stained and fixed with Coomassie Blue R-250 in 10% ethanol and 5% acetic acid, destained, and treated with Amplify (Amersham, Switzerland) for 30 min, dried, and then exposed to Fuji RX medical x-ray films at −80 °C. The percentage of processed human LPH was obtained by quantifying the radioactivity in the dried gels with a PhosphoImager scanner using Imagequant 3.2 software (both from Molecular Dynamics).

Immunofluorescence—Transfected cells grown on glass coverslips were fixed for 30 min with 3% paraformaldehyde in PBS, treated for 15 min with 0.1 M glycine in PBS, and permeabilized or not for 5 min with 0.25% Triton X-100 in PBS. LPH wt and its mutants were detected with the monoclonal anti-human LPH antibody and fluorescein isothiocyanate-conjugated goat anti-mouse secondary antibody (1:100, Tago, Switzerland).

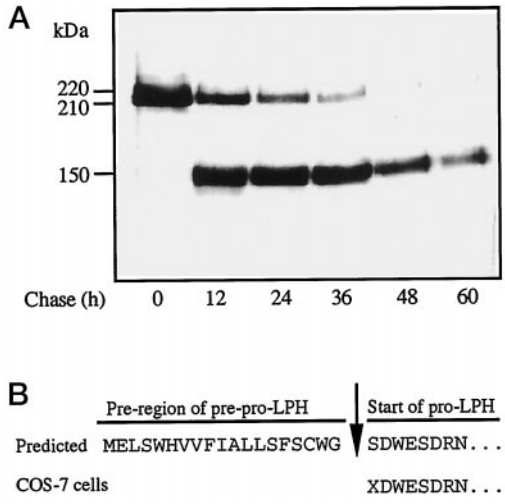

FIG. 1. **Lactase production in COS-7 cells.** *Panel A*, biosynthesis and post-translational processsing of pro-LPH expressed in COS-7 cells. The cells were radiolabeled for 2 h followed by a chase for the time periods indicated. After electrophoresis the dried gel was exposed for autoradiography. 210 kDa = pro-LPH (high mannose form); 220 kDa = pro-LPH (complex-glycosylated form); 150 = mature-like LPH. *Panel B*, NH_2-terminal amino acid sequence (residues 20–27) of pro-LPH produced in transfected COS-7 cells determined by direct amino acid sequencing.

Measurement of Lactase Activity and Protein Determination—COS-7 cells transfected with LPH wt or LPH mutants were detached with a rubber policeman and collected in cold PBS. The cells were disrupted with a conical grinding pestle and sonicated (15 s, 60 watts). Lactase activity was determined as described by Wacker *et al.* (2). Briefly, cell homogenates were incubated at 37 °C for 1 h in 33 mM sodium maleate buffer, pH 6.0, using 33 mM lactose as substrate. After boiling for 2 min the liberated D-glucose was determined at 365 nm with D-glucose dehydrogenase (Merck). Protein concentrations were determined by the method of Bradford (33).

Amino Acid Sequence Analysis of Pro-LPH and Mature-like LPH Forms—Pro-LPH and mature-like LPH were immunopurified from COS-7 transfected cells, separated by gel electrophoresis, and blotted onto a polyvinylidene difluoride membrane. The membrane was washed and stained in 0.1% Coomassie Blue R-250 in 50% methanol. Individual protein bands were cut from the membrane. The NH_2-terminal amino acid sequence of a portion of each band was determined by automated Edman degradation using an Applied Biosystems 470 gas phase Sequencer. When radioactive amino acid sequencing was performed, COS-7 cells cotransfected with prepro-LPH and furin cDNAs were labeled with [^{3}H]phenylalanine for 1 h and chased for 16 h. The immunoprecipitated proteins were separated by SDS-PAGE, then radioactive bands were excised from the polyvinylidene difluoride membrane and used directly for protein sequence determination. Radioactivity in each cycle was determined by collecting the anilinothiazolinone amino acid derivatives and counting in a Beckman LS1801 scintillation counter.

RESULTS

Processing of Human Prepro-LPH and Pro-LPH in Transfected COS-7 Cells—Human prepro-LPH undergoes a number of incompletely described proteolytic cleavage events in the constitutive secretion pathway *in vivo*. To study the processing of human prepro-LPH in detail we used COS-7 cells as a model. This cell line was used successfully in our laboratory to study the processing of rabbit lactase (23). Fig. 1*A* shows that the lactase precursor, immunopurified from transiently transfected COS-7 cells, is processed to a 150-kDa mature or mature-like protein. This processing, however, takes place far more slowly than in enterocytes. COS-7 cells need almost 3 days to process pro-LPH completely to the 150-kDa mature or mature-like protein.

To investigate the processing in detail, we first examined whether cleavage of prepro-LPH by signal peptidase in COS-7

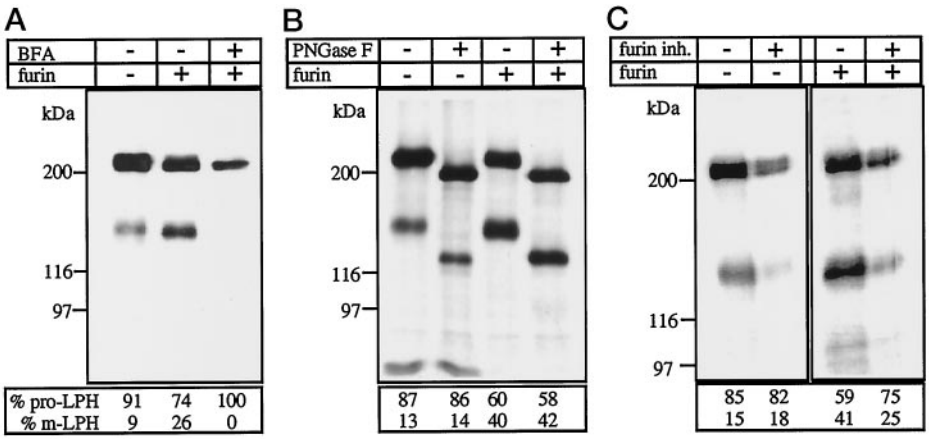

FIG. 2. **Analysis of endoproteolytic processing of human pro-LPH by furin.** *Panel A*, COS-7 cells were transiently transfected either with cDNA coding for human prepro-LPH alone or with cDNAs encoding furin and human prepro-LPH. The cells were metabolically labeled for 1 h and chased for 5 h. BFA was used at a concentration of 5 μg/ml. *mLPH*, mature-like LPH. *Panel B*, COS-7 cells were transiently transfected either with cDNA coding for human prepro-LPH or prepro-LPH together with cDNA coding for furin (chase time: 7 h). After immunoprecipitation the samples were divided into two aliquots, one of which was treated with PNGase F before further processing. *Panel C*, COS-7 cells were transiently transfected either with cDNA coding for human prepro-LPH or prepro-LPH together with cDNA coding for furin. The cells were metabolically labeled for 1 h and chased for 7 h. The furin inhibitor, decanoyl-Arg-Val-Lys-Arg-chloromethyl ketone (*furin inh.*) was used at a concentration of 100 μM in the medium, during both pulse and chase.

cells occurred at the same position as expected in enterocytes (1) and as found in MDCK cells transfected with prepro-LPH cDNA (4). NH_2-terminal amino acid sequence determination of immunopurified pro-LPH confirms that the cleavage takes place after Gly^{19}, *i.e.* where predicted for human prepro-LPH (1) (Fig. 1*B*). However, for the further processing of pro-LPH to mature or mature-like LPH, we failed to obtain an unambiguous NH_2-terminal amino acid sequence of this LPH form (approximately 150 kDa) slowly generated in COS-7 cells, suggesting that pro-LPH is cleaved at more than one site in COS-7 cells.

Generation of Mature-like LPH Form in COS-7 Cells Expressing Furin—We have shown previously that predominantly furin, PC1/3, and/or PC6A are implicated in the processing of rabbit pro-LPH to generate the 180-kDa intermediate form (23). We therefore investigated whether furin generates human mature-like LPH. Furin is localized mainly in the *trans*-Golgi network with ubiquitous tissue distribution (12). Furthermore, it can translocate between the cell surface and the *trans*-Golgi network (34, 35), a behavior that is in agreement with its proposed role in multiple processing events.

To examine the potential of furin to cleave human pro-LPH in detail, COS-7 cells were transfected with the full-length cDNA of prepro-LPH together with the cDNA of furin. Fig. 2*A* shows that furin is able to increase the amount of protein referred to as mature-like LPH approximately 3-fold after 5 h of chase. The apparent molecular mass is very similar to the mass of mature-like lactase generated by endogenous proteases in COS-7 cells (150 kDa). This is more clearly visible after deglycosylation with PNGase F (Fig. 2*B*). Processing is blocked by BFA (Fig. 2*A*), which interrupts transport from the ER to the Golgi. Processing of pro-LPH in the small intestine is known to take place in the *trans*-Golgi network or in a later compartment (31, 36, 37), where furin is also known to be located.

The enhanced processing of pro-LPH by coexpressed furin is inhibited by the membrane-permeant specific inhibitor of furin decanoyl-Arg-Val-Lys-Arg-chloromethyl ketone (29) when added to the medium during the pulse-chase period (Fig. 2*C*). This inhibitor is not able to inhibit processing of lactase by COS-7 cell endogenous proteases (although there is a reduction in the amount of immunoprecipitated LPH forms, the inhibitor does not alter the ratio between the 220-kDa pro-LPH and the 150-kDa mature-like form). The cleavage of human pro-LPH in non-furin-transfected COS-7 cells must thus be caused by other proteases.

Determination of NH_2-terminal Sequence of the Intracellular Mature-like LPH Generated by Furin—The results described in the preceding paragraph show that overexpressed furin elevates the split of pro-LPH to a mature-like protein in COS-7 cells. At least two furin motifs are present in human pro-LPH shortly upstream of the mature NH_2 terminus (*i.e.* at Arg^{832} and $Lys\text{-}Arg^{854}$, Fig. 3*A*). To identify the cleavage site used by furin, COS-7 cells, cotransfected with prepro-LPH and furin cDNA, were labeled with [^{3}H]phenylalanine. The lactase forms were immunoprecipitated, and mature-like lactases were subjected to radioactive amino acid sequencing. The results (Fig. 3*B*) show that human pro-LPH is cleaved by furin after the motif $R\text{-}T\text{-}P\text{-}R^{832}$ (Fig. 3*A*) to generate a NH_2 terminus starting at Lys^{833}. This protein is 36 amino acids longer than the native, mature LPH found in the brush-border membrane (NH_2 terminus, Ala^{869}) (1, 38).

Expression of Prepro-LPH Wild Type and Mutants—To provide further experimental evidence for the cleavage of pro-LPH by furin, we altered the furin motif R-T-P-R by site-directed mutagenesis (mutant pro-LPH R829T/R832T) and generated two additional mutants: pro-LPH K853T/R854T (a dibasic site located between the former and the final NH_2 terminus) and pro-LPH K866T/R868T (thus abolishing the K-V-R motif just in front of the native NH_2 terminus of mature LPH) (Fig. 4*A*). All constructs as well as wt prepro-LPH were expressed in COS-7 cells. The cells were labeled with [^{35}S]methionine for 1 h and chased for 6 h. Immunoprecipitation of LPH wt with the antibody against the mature lactase reveals 210/220-kDa species (the range of sizes of pro-LPH, high mannose and complex glycosylated, respectively) and a weak (10% of total) 150-kDa protein (approximately the size of mature LPH) (Fig. 4*B*). Immunoprecipitations of lactase mutants K853T/R854T and K866T/R868T showed a pattern similar to that of lactase wt. In contrast, immunoprecipitation of mutant pro-LPH R829T/R832T revealed only one polypeptide with an apparent molecular mass of 210 kDa which corresponded to the size of the high mannose form (Fig. 4*B*). In the presence of cotransfected furin we could again observe the furin-mediated enhanced processing of wt pro-LPH as well as the enhanced processing of the mutants K853T/R854T and K866T/R868T. However, in the presence of coexpressed furin the mutant R829T/R832T is not processed (Fig. 4*B*). Similar results were obtained with LoVo and Ltk^- cells (data not shown).

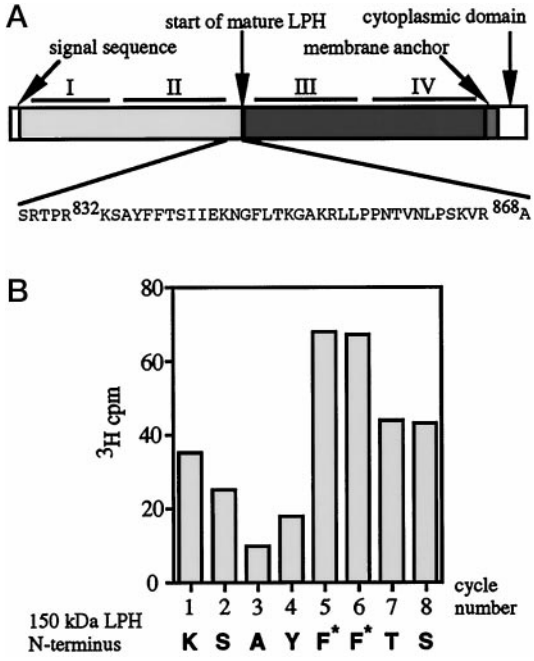

FIG. 3. **NH_2-terminal sequence analysis of the radiolabeled mature-like LPH produced in COS-7 cells cotransfected with furin.** *Panel A*, diagram of human LPH as predicted from the cDNA sequence (1) and the amino acid sequence encompassing the last amino acid of the pro domain (Arg^{868}) and and the furin motif (Arg^{832}) 36 amino acids upstream. *Panel B*, COS-7 cells were transiently transfected with cDNAs coding for human prepro-LPH and furin. They were metabolically labeled for 1 h in medium containing [^{3}H]phenylalanine and then chased for 10 h. The radioactive band corresponding to mature-like LPH was excised and used directly for sequence determination. The *bars* show the radioactivity released in each cycle. The predicted NH_2-terminal sequence of the mature-like 150-kDa LPH based on processing at the COOH-terminal site of the R-T-P-R^{832} furin cleavage site is shown below. * indicates the phenylalanine residues predicted to be radioactive in the NH_2-terminal sequence resulting from cleavage at Arg^{832}.

To examine the fate of this mutant R829T/R832T two parameters were studied: lactase enzymatic activity and cellular localization of this mutant. Fig. 4*C* (*white bars*) shows that lactase activities of wt and the mutant proteins K853T/R854T and K866T/R868T are clearly measurable (although the activity of mutant K866T/R868T is somewhat lower). Mutant R829T/R832T, in contrast, does not display detectable lactase activity. Similar results were obtained in the presence of furin (Fig. 4*C*, *black bars*). Because the enzymatic activity of pro-LPH is known to be the same as that of mature LPH (39), the absence of activity cannot simply be attributed to incomplete processing of the pro-LPH mutant R829T/R832T. We tested whether mutant R829T/R832T had reached the plasma membrane. COS-7 cells were transfected with wt or mutant cDNAs of prepro-LPH. 48 h later, the cells were fixed and either permeabilized with Triton X-100 or not permeabilized. Indirect immunofluorescence was used to localize the lactase forms. Pro-LPH wt (*a*) and mutants K853T/R854T (*c*) and K866T/R868T (*d*) are found on the cell surface and also in intracellular compartments (probably the ER and Golgi) (Fig. 5). Mutant R829T/R832T (*b*), however, is detectable only after cell permeabilization and is localized around the nucleus, *i.e.* it is retained intracellularly. This observation is in agreement with the result shown in Fig. 4*B* for this mutation, where only the 210-kDa precursor, indicative of high mannose pro-LPH, was expressed. The same immunolocalization of pro-LPH wt and mutants was found in cells cotransfected with furin (data not shown).

Further Processing of Mature-like LPH —It is known that the treatment of pro-LPH molecules with trypsin alone generates a lactase with an apparent molecular mass similar to that of brush-border mature LPH (39). Thus, it appears likely that subsequent cleavage of human mature-like LPH by trypsin leads to the generation of the "mature" enzyme *in vivo*. To provide evidence for this assumption, we incubated cell extracts from COS-7 cotransfected with or without furin with trypsin and analyzed the proteins by SDS-PAGE before and after deglycosylation with PNGase F (Fig. 6). Deglycosylated mature-like LPH treated with trypsin has an apparent molecular mass of 120 kDa (corresponding to the calculated mass of 120,993 Da of mature human intestinal lactase). Deglycosylated mature-like LPH not treated with trypsin has an apparent molecular mass of approximately 124 kDa. This small shift in the molecular mass of mature-like LPH produced by COS-7 cells is independent of the presence or absence of overexpressed furin, indicating again that endogenous COS-7 proteases cleave pro-LPH very close to or at the furin motif around Arg^{832}.

These results suggest that the brush-border mature LPH is most probably produced by a multistep processing involving, *e.g.* furin, perhaps more PC(s), aminopeptidases, granzyme A (52), and/or pancreatic proteases (*e.g.* trypsin).

DISCUSSION

Intestinal human lactase is synthesized as a large precursor (prepro-LPH) of 1927 amino acid residues which is subjected to a number of proteolytic and other events before (and probably also after) reaching the final destination, the brush-border membrane. As prepared from this membrane, human LPH is a glycoprotein with an apparent molecular mass (by SDS-PAGE) of approximately 150–160 kDa, encompassing the amino acid sequence from Ala^{869} to the COOH terminus (calculated molecular mass of the polypeptide chain, 120,993 Da). Mature brush-border LPH corresponds, therefore, to the COOH-terminal 56% of the original sequence of prepro-LPH. Although number, types, and cellular sites of the proteolytic events producing mature LPH from prepro-LPH have been studied extensively, albeit with controversial results, much less has been learned about the proteases implicated in the maturation. A central question in our investigation was whether PCs of the furin type may partake in the series of events forming mature LPH from human pro-LPH, as we have shown for the 180-kDa intermediate occurring in the rabbit (23).

Processing of Prepro-LPH to Pro-LPH—To make sure that the pro-LPH expressed in COS-7 cells had the same NH_2 terminus as pro-LPH formed in enterocytes, we have verified that prepro-LPH is correctly converted to pro-LPH. Clearly (Fig. 1*B*), the signal peptidase in COS-7 ER splits prepro-LPH at the site predicted for the human, *i.e.* after Gly^{19} (1) and found in MDCK cells transfected with human prepro-LPH cDNA (4). Hence, human pro-LPH expressed in COS-7 cells has the same amino acid sequence as that expected in enterocytic pro-LPH.

The First Step of Processing Human Pro-LPH—Previous reports from various laboratories, including ours, have indicated that COS cells do not detectably process human pro-LPH (4, 23, 25, 39). In all of these studies fairly short chase times of a few hours or less were used. We now find that long chase periods of 12–60 h do lead to substantial processing of pro-LPH to a mature-like LPH by endogenous proteases of COS-7 cells. The need for long chase periods can be attributed to a number of reasons, alone or in combination: COS-7 cells may express PCs in insufficient amounts to process the overexpressed amounts of LPH rapidly, or the endogenous proteases have a subtly different substrate specificity; COS-7 cell-specific glycosylation may cause steric hindrance and/or subtly alter the structure of pro-LPH segments otherwise susceptible to the action of PCs. Because the mature-like LPH proteins obtained after extended chase periods did not reveal unambiguous NH_2-terminal sequences we have at least indirect evidence for the assumption

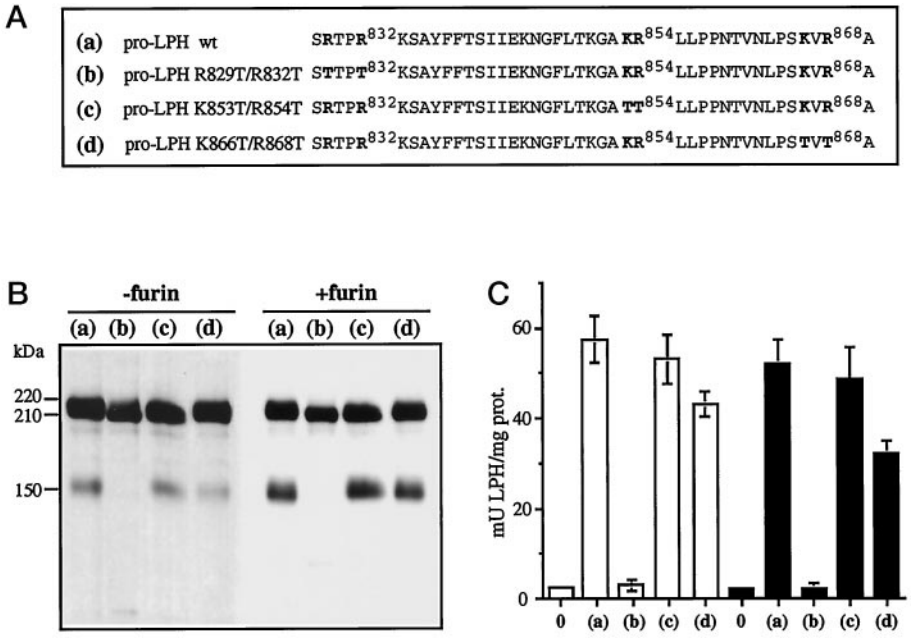

FIG. 4. **Processing of pro-LPH wt and pro-LPH mutants.** *Panel A*, location of the mutations in the pro sequence: (*a*) pro-LPH wt, (*b*) pro-LPH mutant R829T/R832T, (*c*) pro-LPH mutant K853T/R854T, (*d*) pro-LPH mutant K866T/R868T. *Panel B*, analysis of endoproteolytic processing of human pro-LPH wt and pro-LPH mutant proteins in COS-7 cells. Cells were transfected with the corresponding cDNA of prepro-LPH wt (*a*) and its mutants (*b*, *c*, and *d*) with or without furin, labeled for 1 h, and chased for 6 h. (Only exposure of gels for shorter times permits clear distinction of the two bands at 210 and 220 kDa but does not show the mature-like form any longer (150 kDa).) *Panel C*, *white bars*, lactase enzymatic activity of the different lactases: wt (*a*) and mutants (*b*, *c*, and *d*) expressed in COS-7 cells was compared with lactase activity of untransfected cells (*0*). *Black bars*, the activity of different LPH forms cotransfected together with furin cDNA. The results are the mean of three independent experiments ± S.E.

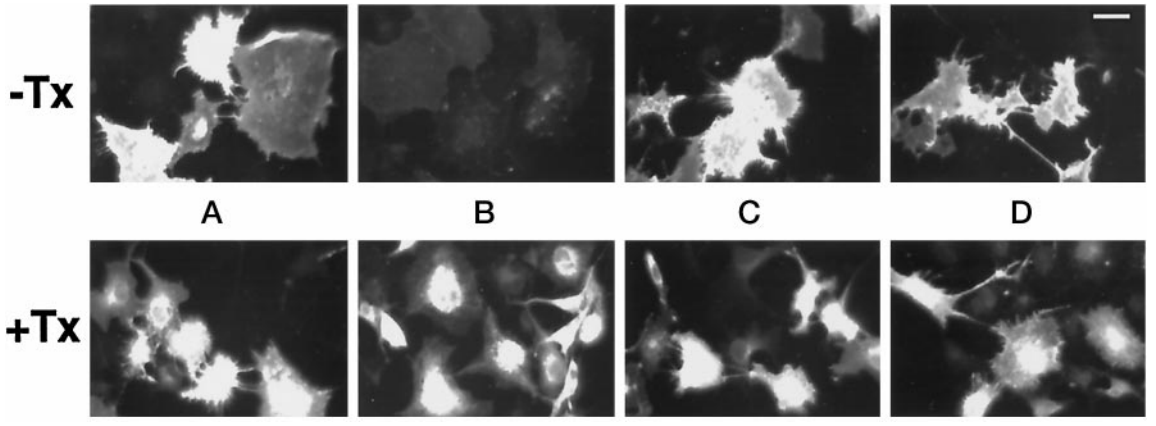

FIG. 5. **Immunolocalization of pro-LPH wt and pro-LPH mutants in COS-7.** Plasmids coding for prepro-LPH wt (*A*), R829T/R832T mutant (*B*), K853T/R854T mutant (*C*), and K866T/R868T mutant (*D*) were introduced into COS-7 cells. 48 h after transfection the cells were fixed and either permeabilized with Triton X-100 (+*Tx*) or not permeabilized (−*Tx*). The pro-LPHs were detected by indirect immunofluorescence using the monoclonal antibody against mature human LPH. *Bar* = 20 μm.

that more than one type of protease is implicated in the processing of pro-LPH overexpressed in COS-7 cells. The relative apparent homogeneity of the protein band (see Fig. 1*A*), however, strongly suggests that these cleavages occur within a small segment of pro-LPH. From the data in Fig. 1*A* a precursor/product relationship is not obvious; but this can be attributed to the short lived protein production in transient expression assays and the long chase periods.

The size of mature-like LPH formed in COS-7 and in MDCK cells by endogenous proteases (24) is very similar to the size of mature brush-border LPH, pointing to a proteolytic cleavage taking place only very few kDa upstream of the NH_2 terminus of mature, brush-border LPH. In the cDNA-derived sequence of prepro-LPH some motifs for furin-like PCs do occur at a short distance from the native NH_2 terminus, in particular one, 36 amino acids (or 4,034 Da) upstream of the native NH_2 terminus, R-T-P-R^{832}. Overexpression of prepro-LPH together with furin in COS-7 cells led to a 3-fold increase in the amount of mature-like LPH produced by these cells. The addition of decanoyl-Arg-Val-Lys-Arg-chloromethyl ketone, a reasonably membrane-permeant furin inhibitor (29), to the medium inhibited the enhanced processing of pro-LPH to mature-like LPH (Fig. 2*C*). Adding BFA to the medium also blocked this processing, and furin is located both in the Golgi apparatus and at the cell surface and translocates between these compartments (34, 35). Finally, the site of this split in COS-7 cells cotransfected with prepro-LPH and furin was identified unequivocally by radioactive NH_2-terminal sequence analysis of mature-like LPH band using [^{3}H]phenylalanine: the resulting sequence *X-X-X*-F-F-*X-X* was in perfect agreement with the expected site of processing by furin between positions 832 and 833, *i.e.* after the motif R-T-P-R^{832} (Fig. 3). There can be little doubt, therefore, that this processing is indeed caused by furin.

Jacob *et al.* (24) also reported the occurrence of an intermediate in the processing of human pro-LPH in human small intestinal biopsies or in transfected MDCK cells. Their intermediate is also 3–4 kDa longer than mature LPH produced *in vitro* by the addition of trypsin after biosynthesis. Contrary to these and our own observations (previous paragraph), the same group (4) has, however, also reported that a lactase form arises from cleavage between positions Arg^{734} and Leu^{735} (not a furin motif) in MDCK cells; this form is thus 134 amino acid residues longer (*i.e.* larger by 15,132 Da) than mature, brush-border LPH, which makes it a different and unlikely candidate as an intermediate in the processing of pro-LPH to mature LPH in enterocytes. It could perhaps arise from the action of lysosomal proteases to which pro-LPH

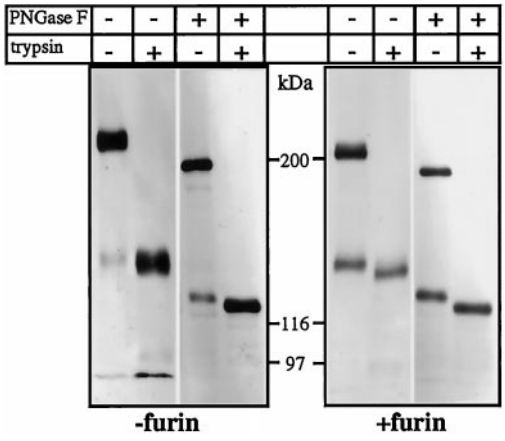

FIG. 6. **Trypsin treatment of detergent extracts from COS-7 cells.** Prepro-LPH was expressed in COS-7 cells with or without furin. The cells were metabolically labeled for 1 h and chased for 7 h. Then cell lysates were prepared, treated with 50 μg of trypsin for 5 min at 37 °C, and immunoprecipitated with the monoclonal antibody against human LPH. Each sample was divided into two equal aliquots, one of which was treated with PNGase F.

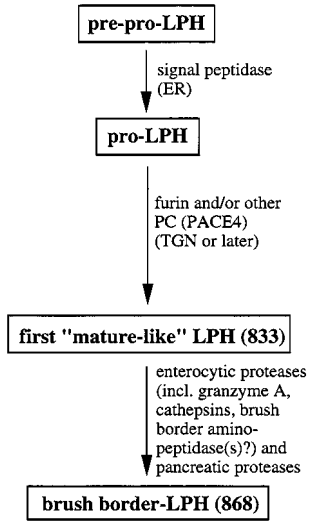

FIG. 7. **Schematic representation of the proteolytic processing of human prepro-LPH.** In the ER prepro-LPH is cleaved by signal peptidase to generate pro-LPH, which is then cleaved in the *trans*-Golgi network by a PC (furin or furin-like) to generate an intermediate mature-like LPH. form The mature-like LPH is targeted to the brush-border membrane (either directly or while undergoing additional proteolytic cleavage(s) by endoproteases and/or aminopeptidases), in which it is processed by pancreatic proteases (*e.g.* trypsin) to yield the final mature LPH form. The *numbers* in *parentheses* refer to the NH_2-terminal amino acid number in the respective form.

expressed in COS-1 appears to be exposed (40).

The Double Mutant R829T/R832T of Human Pro-LPH Fails to Reach the Plasma Membrane of COS-7 Cells—As discussed above, furin, coexpressed with prepro-LPH in COS-7 cells, splits pro-LPH at the sequence R-T-P-R^{832}. We searched for further confirmation of this conclusion by modifying this and other consensus sequences by site-directed mutagenesis. Although this experiment did not contribute to new conclusions, it turned out to be interesting. Unexpectedly, the double mutant R829T/R832T failed to appear in the plasma membranes of these cells; it was retained in paranuclear bodies, tentatively identified as ER (Fig. 5*B*). Thus, although no mature-like LPH arose from this mutant, no conclusion could be drawn as to whether the mutant was or was not susceptible to the action of furin (which is located in later cell compartments). Probably this mutant (and similar ones generated by others (24)) did not acquire a conformation necessary to proceed past the ER. A mechanism involving an abnormal conformation of the pro sequence is appealing because it correlates with another observation: the pro sequence must be linked to the rest of LPH to guarantee transport to the plasma membrane (25, 41): "pre-LPH" (*i.e.* a lactase expressed with the signal peptide but devoid of the pro segment) is retained in paranuclear organelles. The simultaneous formation of the pro domain and of pre-LPH as separate individual proteins does not allow LPH to exit from the ER, suggesting that the pro domain in its proper context (and therefore properly folded) is essential for pro-LPH transport to the plasma membrane.

It is known that proteins synthesized in the ER have to form homo- or hetero-oligomers to move past this compartment (42, 43). The mechanism explaining why the pro domain in pro-LPH is needed for pro-LPH to reach the plasma membrane is not known, but oligomerization has been suggested (25, 41, 44–46). Alternatively, the pro domain might protect the lactase proper from being cleaved by intracellular proteases before reaching the plasma membrane. Whatever the mechanism, a correctly folded pro region is needed, although some changes may be tolerated (*e.g.* in the double mutants in Fig. 4, *c* and *d*, and Fig. 5, *C* and *D*). For some reason the double mutation R829T/R832T interferes with this process (the lack of two positive charges may impinge on the folding and/or on interactions).

Comparison of the four internal repeats I–IV in pro-LPH reveals that furin-like motifs are present in mature LPH at locations germane to Arg^{829} and Arg^{832}, both in repeat III (Arg^{1346} and Arg^{1349}) and in repeat IV (Arg^{1822} and Lys^{1825}), *i.e.* in mature lactase. Whether this is of relevance or not is unclear at this moment; at any rate, none of the "furin motifs" in repeats III and IV seems to be attacked by any of the PCs, although their sequences, R-T-A-R and R-I-P-K, would be in principle susceptible to attack by furin-like PCs (47).

Summing Up—we have shown that pro-LPH arising from prepro-LPH in COS-7 cells transfected with the corresponding human cDNA has the same NH_2 terminus expected in pro-LPH from human enterocytes; that the proteolytic processing of this pro-LPH is blocked by BFA (like the pro-LPH in cultures of human small intestine (31), showing that this processing occurs in the Golgi or in a later compartment (where a number of proteases has been localized, including furin and furin-like PCs); that COS-7 cells cotransfected with prepro-LPH and furin cDNA process pro-LPH to a mature-like LPH, some 3–4 kDa larger than mature LPH; that this processing is inhibited by a membrane-permeant furin inhibitor; and that this processing takes place at position R-T-P-R^{832}, which is one of the furin sequences (47). This cleavage site is not far from but not identical with that suggested by others (24) (who, incidentally, used a somewhat different numbering). Although the work in the present paper was carried out with transfected COS cells, our results make it most likely that one or more of these PCs contribute to the processing of human pro-LPH in the enterocytes *in vivo*, because furin and furin-like PCs do occur in enterocytes (23).

The proteolytic processing of pro-LPH to LPH in man and in the rabbit appears to involve in both species one or more furin-like PCs. The most conspicuous difference is the apparent size of the first intermediate, which is approximately 180 kDa in the rabbit (23) but only 3–4 kDa larger than mature LPH in man (mature-like LPH). The furin sequences cleaved in rabbit or in the human pro-LPH are not the same. Rabbit pro-LPH is cleaved at R-A-A-R^{349} in the second half of the homologous region I (50); human pro-LPH does not have a furin motif here. Human pro-LPH is split, instead, as shown here, to a far smaller first intermediate, at the sequence R-T-P-R^{832}, which occurs in the rabbit also but is not utilized (50). (More furin sequences occur in

the pro sequences of pro-LPH, but a few only are utilized.)

How Is Mature, Brush-border LPH Ultimately Generated in Vivo?—The NH_2-terminal sequence in mature, brush-border LPH is A-F-T-F-P . . . in man (38), A-S-A-L-P . . . in rabbit (1), and V-T-D-S-L . . . in rat (48). As deduced from the cognate cDNAs, these NH_2-terminal sequences are preceded in pro-LPH, by the amino acids P-S-K-V-R- (human (1)), P-S-K-T-R- (rabbit (1)), and T-S-R-A-R- (rat (48)). An apparent consensus motif (K/R-*X*-R-) immediately upstream of the NH_2 terminus of mature LPH could thus be made out. Wüthrich *et al.* (4) mutated the Arg^{868} in human pro-LPH (which immediately precedes the NH_2-terminal Ala^{869} in mature, brush-border LPH) to a Ile, Lys, or Glu; this had no effect on the proteolytic processing of pro-LPH to mature (or rather mature-like) LPH in transfected Caco-2 cells (as judged by SDS-PAGE). Similar observations have been reported for another mutant also, in which Arg^{868} was changed into a Thr and expressed in MDCK cells (24). This mutant was not split by trypsin. Clearly, the proteolytic processing that leads to mature, brush-border LPH is more complicated than originally expected.

The events following the furin split after Arg^{832} and eventually removing the amino acids up to position 869 (the NH_2 terminus of mature, brush-border LPH) are still poorly understood. The most straightforward mechanism, as suggested, *e.g.*., by Jacob *et al.* (24) for their intermediate is that trypsin simply splits mature-like LPH after Arg^{868}, thereby releasing a 36-amino acid long peptide. This mechanism would in fact be very plausible and agrees with our observation that both in the presence and absence of overexpressed furin cDNA human mature-like pro-LPH is cleaved by trypsin to its final form. However, the brush-border LPH from the enterocytes of (rat) Thiry-Vella loops (which has never been exposed *in vivo* to pancreatic proteases) is 2 amino acids longer than "regular" rat LPH, *i.e.* has an NH_2-terminal sequence A-R-V-T-D-S-L . . . , rather than V-T-D-S-L . . . (51). The enterocytes can, therefore, almost (but not quite) produce mature, brush-border LPH in the absence of trypsin. As mentioned in the previous paragraph, the amino acid sequences immediately upstream of A-R-V-T-D-S-L . . . are very homologous in rat rabbit and man. It seems likely, therefore, that they are removed in the three species by the same or similar proteases before the final trimming of the last two amino acid residues by luminal trypsin. In other words, it is most likely that the 34 amino acids between Arg^{832} and Lys^{866} (see *sequence a* in fig. 4*A*) are removed as small peptides and/or individually by enterocytic proteases, possibly including granzyme A (52), brush-border aminopeptidases, and perhaps lysosomal cathepsins (Golgi resident proteins have been shown to be exposed to cathepsins (49) and (pro)lactase itself is likely to be exposed to lysosomal proteolysis in COS-1 cells (40)).

In conclusion, human pro-LPH, far from being processed to mature LPH in a single step, is subjected to a first intracellular, furin-like cut at position Arg^{832} and then to further cuts, by enterocytic and (probably at the very end) by pancreatic proteases (Fig. 7). The chain of events identified in the present work provides therefore a more detailed picture to the concept, already put forward earlier (see *e.g.* 53, 54), that in the processing of pro-LPH pancreatic proteases do play a role, but a fairly limited one.

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Processing of Human Intestinal Prolactase to an Intermediate Form by Furin or by a Furin-like Proprotein Convertase

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